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MUTAGENIC AND GENOTOXIC SCREENING OF EIGHT COMMONLY USED SKIN WHITENING CREAMS IN NIGERIA

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ABSTRACT
Skin whitening (bleaching) creams are often used to deliberately lighten the skin in response to social pressures or for the treatment of skin pigmentation. Bleaching creams contain varied concentrations of hydroquinone, corticosteroids, ammoniated mercury and kojic acid. Prolonged use of these creams may have deleterious (mutagenic) effect on the genetic material (DNA) of body cells. The purpose of this study was to evaluate the mutagenicity potentials of eight commonly used bleaching creams (Body white, Tura, Maxitone, Movate, Amos white, Top gel, Ultra clair, Fair and white), using the modified Ames test (with and without metabolic activation) that uses the wild type Escherichia coli (0157:H7) as tester strain. The assay was examined for revertant strains of the organism with at least three alterations in the phenotypic characteristics of the wild type organism. Results obtained showed that the eight bleaching creams produced revertant strains of the organism with alteration in more than three of its phenotypic characteristics and compared favourably with the standard mutagen (ethidium bromide), which produced the same effect. Three mutation mechanisms (forward, backward and silent mutations) were identified. The introduction of liver enzymes (S9 mix) made no significant difference in the number of characteristics altered ($p>0.05$). The results of this study revealed that the eight bleaching creams were mutagenic in bacteria and could be said to possess carcinogenic potentials. Their mechanism of mutagenesis could also be by intercalation just as ethidium bromide.

Key words: Bleaching cream, Ames test, revertant strain, mutagenicity, intercalating mutagen.

INTRODUCTION

The use of skin lightening (bleaching) creams is on the increase not only by women but also men who often initiate the practice either for the treatment of skin pigmentation caused by acne vulgaris, eczema, atopic dermatitis, ptyrasis rosea or to deliberately lighten the skin in response to social pressures (Mahe *et al*., 1994). The use of bleaching creams cut across all sociodemographic characteristics. The habit of bleaching the skin is most rampant among commercial sex workers who camouflage their occupation in the clinic data as "fashion designer" because of the opprobrium attached to prostitution (Olumide *et al*., 2008). Skin lightening creams are prescribed in clinics by physicians as therapeutic treatment for hyperpigmentation of the skin such as melasma, chloasma, solar lentigenes or that may occur during pregnancy (Diven *et al*., 1990). They also conceal age spots, freckles or skin pigmentation that may occur from exposure to the sun. An additional application is genital or anal bleaching intended to reduce the typically darker pigmentation of the genital and perianal areas.

Complications from the use of skin lightening creams have raised serious concern. Studies have been conducted on the general effects of some of the ingredients used in the making of these creams. Some of these ingredients have depigmentation properties and when used persistently for long periods of time can result in the building up of concentrations that can be deleterious to body cells. Hydroquinone has been reported to be a potential mutagen (Nigam, 2009). Reports from Europe and United States documented exogenous ochronosis in people who have used creams containing 2% and even 1% hydroquinone (Cullison *et al*., 1983; Lawrence *et al*., 1988; Diven *et al*., 1990). Nephrotic syndrome due to topical or systemic use of mercury has been well documented (Silverberg *et al*., 1967; Barr *et al*., 1972). The introduction of topical steroids has caused harms such as irritation and mild burning sensations in humans (Lyubojeviae *et al*., 2002; Rathi, 2006).

The situation is even more worrisome for dark skinned people due to the protective functions of the black skin against the damaging effects of UV irradiation which could cause sunburn and skin cancers for exposed vulnerable skins (Lin and Fisher, 2007).Other complications that arise from skin bleaching include contact dermatitis, contact leucoderma, nephrotic syndrome, acute toxicity, folliculitis, skin fragility, atrophy of the skin and predisposition to cutaneous infections (Fisher, 1994; Olumide *et al*., 2008). Despite the fact that researchers have written a lot concerning the inherent hazard which bleaching creams pose for the users, their use has continued unabated. Bleaching creams act at various levels of melanin production in the skin. As a result of its critical role in melanin biosynthesis, the enzyme tyrosinase has become a major target for inhibition in several ways in bleaching creams (Nico *et al*., 2009). Some chemical ingredients in bleaching creams can have effect on the genetic make up of the cell and lead to development of cancerous cells in users.

There have been reports of the existence of mutagenic chemicals (including newly synthesized ones) in our environment (Frederick, 1978). In order to evaluate the mutagenic potentials of this diverse entity, tests and screening procedures have been developed. Mutagenic potentials when ascertained act as an index of carcinogenicity and genotoxicity. Ames *et al.* (1975) reported a 90% correlation between mutagenicity and carcinogenicity. Development of mutagenicity test methods is important in order to protect public health. An array of short-term mutagenicity test systems have been developed employing both prokaryotic and eukaryotic organisms. Above all, the test developed by Ames *et al*. (1975) (popularly known as Ames test) has been the most widely used. The popular Ames test lacks qualities of a good and rapid test system which are sensitivity, rapidity, inexpensiveness and simplicity. Therefore, alternative short-term mutagenicity bacterial tests which are able to measure other endpoints are needed. In this regard, a modification of Ames test which relies on changes in at least three biochemical characteristics of wild type *E. coli* (0157:H7) as an index for mutagenicity was developed by Akintonwa *et al.* (2007).

Using this principle, potassium bromate, two pharmaceutical effluents, some medicinal plants, petrol and engine oil have been reported to have mutagenic activities (Akintonwa *et al.*, 2007; Akintonwa *et al.*, 2009a and b; Awodele *et al.*, 2010). Akintonwa *et al.* (2008), on the contrary reported that some brands of commonly used insecticides (Baygon, Mobil, Mortein and Total) were not mutagenic in the same bacterial system.

The purpose of this research therefore, was to assess the mutagenicity and hence carcinogenicity of eight bleaching creams (Body white, Tura, Maxitone, Movate, Amos white, Top gel, Ultra clair and Fair and white) using the modified Ames test. An attempt was also made to elucidate the exact mechanism of mutagenesis of each of the bleaching creams.

MATERIALS AND METHODS

Bacterial Strain

Wild type *Escherichia coli* (0157:H7) strain obtained from Lahor Research Laboratories, Benin City, was used. The cultural and biochemical characteristics were re-confirmed as lactose fermenting, motile, urease negative, indole positive and citrate negative. Purified single colony isolates were sub-cultured on nutrient agar slants and stored at 4^0C.

Media and Biochemical

MacConkey agar, Simmon citrate agar, Brain heart infusion agar, Brain heart infusion broth, Nutrient agar (Oxoid) and Christensen's urea broth were prepared according to the manufacturer's direction. All biochemicals (ethidium bromide and tween 80) were obtained from Lahor Research Laboratories, Benin City.

The Bleaching Creams

The eight bleaching creams used were Body white, Tura, Maxitone, Movate, Amos white, Top gel, Ultra clair, Fair and white. They are commonly used by Nigerians. Table 1 shows the chemical composition of the creams as indicated on the cream containers by the manufacturers. They were purchased from a local market in Benin City. Stock solution of each cream was prepared by mixing 25mg of cream with 100ml of 25% tween 80. They were stored at 4^0C. This gave a stock concentration of 0.25mg/ml.

Minimum Inhibitory Concentration (MIC) Determination

The MIC of the bleaching creams against the bacterial strain was determined by the agar–dilution method previously described by Obaseiki-Ebor (1984). Wet-dried agar plates containing various concentrations of cream (0.25 – 4mg/ml) were inoculated with about $10^5 – 10^6$ cfu of the washed tested culture. The MIC was the lowest concentration of cream that completely inhibited growth after 24h incubation at 37^0C.

Preparation of the Rat Microsomal Liver Enzymes

Three Sprague-Dawley rats weighing 120-150g were obtained from the Animal House of Biochemistry Department, University of Benin, Benin City. The levels of microsomal enzymes (alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase) were determined before and after inoculation using the method of Schmidt and Schmidt (1963). The rats were injected intra-peritoneally with 5mg/kg of phenobarbitone for four consecutive days to induce the liver microsomal enzymes as suggested by Maron and Ames (1984). On the fifth day, the animals were sacrificed and the livers were aseptically macerated using a sterile mortar and pestle. To every 1g of the macerated liver, 5ml of 1.65M KCl solution was added. The homogenate was centrifuged (1200 revolutions min^{-1}) and the supernatant filtered using a sterile membrane filter to obtain the rat microsomal enzyme.

The S9 Mix

The S9 mix was freshly prepared using the method of Maron and Ames (1984). Twenty (20) ml of S9 mix contained 2mls of rat liver enzyme, 10ml of 0.2M phosphate buffer at pH of 7.4, 5.6ml of distilled water, 1ml of 80mM NADP sodium salt hydrate, 1ml of 120mM glucose –6– phosphate and 0.4ml of potassium and magnesium salts solution. The mixture was properly stirred before the addition of the rat liver enzyme.

Bacteria Mutation Assay

This was carried out (with and without metabolic activation) using a modification of the standard Ames test as described by Akintonwa *et al.* (2007). The fraction of the liver enzyme (S9) was used at a concentration of 10% (v/v) in the S9 mix. The effect of the bleaching creams on the tester stain (without metabolic activation) was first investigated by mixing 0.1ml of overnight culture of tester strain with 0.1ml of test agent and ethidium bromide (positive control).

To test the effect of the bleaching creams in the presence of metabolic activation, 0.1ml of an overnight culture of the tester strain in brain heart infusion broth was mixed with 0.5ml S9 mix and 0.1ml of each of the bleaching creams. The mixture was incubated at 37^0C for 24h and later seeded onto brain heart infusion agar plates, while the other portion without S9 mix was also seeded on another set of brain heart infusion agar plates. The plates were incubated at 37^0C for 72h. Revertant strains produced were inoculated onto MacConkey agar and incubated at 37^0C for 24 hours to obtain pure colonies. Thereafter, their biochemical and morphological characteristics were re-examined using the method of Cowan and Steel (1973). An alteration in at least 3 (out of 9) biochemical characteristics was taken as a bench mark for mutagenicity. Alteration in less than 3 of the biochemical characteristics indicated either a weak or non-mutagenic substance.

Statistical Analysis

The chi-square goodness of fit test adopted from Ogbeibu (2005) was used to test for significant differences in the values obtained. All statistical tests were carried out using the SPSS 16.0 Windows based program.

RESULTS

Table 2 shows the minimum inhibitory concentration (MIC) of the eight bleaching creams against the bacterial tester strain with ethidium bromide acting as the positive control. Ethidium bromide, Movate and Amos white inhibited total bacterial growth at the lowest concentration of 1mg/ml. This was followed by Body white and Fair and white creams, both of which inhibited bacterial growth at 2mg/ml. Tura, Maxitone, Top gel and Ultra clair inhibited bacterial growth at the highest concentration of 4mg/ml.

The morphological and biochemical characteristics of wild type *E. coli* (0157:H7) and revertant colonies obtained when treated with each of the eight bleaching creams in the absence and presence of metabolic activation is presented in Table 3 with ethidium bromide acting as positive control. Revertant colonies with as many as 6 (out of 9) alterations in biochemical characteristics were observed with almost all the bleaching creams. All revertant colonies (except Ultra clair) became negative to indole, methyl red, serology and motility unlike the wild type. Lactose fermentation ability was retained in Tura (TR1), Ultra clair (UR1) and Top gel (TGR1) revertant colonies; changed to late lactose fermentation in Maxitone (MR1) and Fair and white (FR1) and lost completely in Body white (BR1), Amos white (AR1) and Movate (MVR1). In their growth parameters, treatment with Body white, Amos white and Fair and white gave same growth inhibition as the positive control. There was almost a total inhibition of growth of the wild type in each of these three cases. Treatment with Maxitone gave no inhibition of growth.

There was no significant difference in the number of characteristics altered with the introduction of S9 mix ($p>0.05$). However, its introduction caused a change in TR2, UR2, TGR2 (from scanty growth to full growth) and from scanty growth to complete absence of growth in BR2, AR2 and FR2. MVR2 had scanty growth while MR2 retained its full growth.

Table 1: Some depigmenting ingredients contained in the bleaching creams

Creams	Ingredients
Body white	Kojic acid, hydroquinone, triethanolamine, methylparaben and propylparaben
Tura	Hydroquinone, mulberry, octyldimethyl PABA
Maxitone	Alpha hydroxyacid (AHA), methylparaben
Movate	Clobetasol propionate, propylen glycol
Amos white	Clobetasol propionate
Top gel	Fluciononide, propylene glycol, triethanolamine
Ultra clair	Hydroquinone. kojic acid, methylparaben and triethanolamine
Fair and white	1 – 4 benzenediol (hydroquinone), triethanolamine, methylparaben, propylparaben

Table 2: Minimum inhibitory concentration (MIC) of the bleaching creams and ethidium bromide

	Concentration (mg/ml)				
Creams	**4**	**2**	**1**	**0.5**	**0.25**
Body white	–	(– +)	+	+	+ +
Tura	(– +)	+	+	+ +	+ +
Maxitone	(– +)	+	+	+ +	+ +
Movate	–	–	(– +)	+	+ +
Amos white	–	–	(– +)	+	+ +
Top gel	(– +)	+	+	+ +	+ +
Ultra clair	(– +)	+	+	+ +	+ +
Fair and white	–	(– +)	+	+	+ +
Ethidium bromide	–	–	(– +)	+	+ +

Key: - : no growth, (– +): scanty growth, +: growth, + +: high growth

Table 3: Characteristics revertant strains in the absence and presence of S9 mix

X	***WT***	***Revertant colonies***																	
		Body white		**Tura**		**Maxi tone**		**Movate**		**Amos white**		**Top gel**		**Ultra clair**		**Fair & white**		**Ethidium bromide**	
		BR1	**BR2**	**TR1**	**TR2**	**MR1**	**MR2**	**MVR1**	**MVR2**	**AR1**	**AR 2**	**TGR1**	**TGR2**	**UR1**	**UR2**	**FR1**	**FR2**	**ER1**	**ER2**
Growth	+	(– +)	–	(– +)	+	+	+	(– +)	(– +)	(– +)	–	(– +)	+	(– +)	+	(– +)	–	(– +)	–
Lactose	+	–	–	+	LLF	LLF	LLF	–	–	–	–	+	LLF	+	–	LLF	–	LLF	–
Indole	+	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	+	–
Urease	–	–	–	+	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–
Citrate	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Methyl Red	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Serotype	+	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
Grams Rxn	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Motility	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
TNC		6	6	6	5	5	5	6	7	6	6	5	5	3	5	6	6	5	6

Key: X = Characteristics, WT = wild type 1: absence of S9 mix, 2: presence of S9 mix, BR: Body white revertant, TR: Tura revertant, MR: Maxitone revertant, MVR: Movate revertant, AR: Amos white revertant, TGR: Top gel revertant, UR: Ultra clair revertant, FR: Fair and white revertant, TNC: Total number of characteristics altered, LLF: Late lactose fermentation, +: Growth/positive reaction, (– +):Scanty growth/reaction, –: No growth/negative reaction .

DISCUSSION

Escherichia coli (0157:H7) was obtained from Lahor Research Laboratories, Benin City. The organism was subjected to the method of identification of *Enterobacteriaceae* as described by Cowan and Steel (1973). The organism was found to have the phenotypic characteristics of lactose fermentation, motile, urease negative, indole positive and citrate negative. The Ames test was modified on the assumption that phenotypic expression is always a function of genotype and the environment (Tixier – Boichard, 2002; Kammenga, 2007). Therefore, any alteration in the above mentioned normal biochemical characteristics of the organism may be due to alteration of the genetic material (DNA). Based on the above assumption, this study set a bench mark for mutagenicity to be any agent that altered at least three (3) normal biochemical characteristics of the organism.

This study used the modified Ames test (Akintonwa *et al*., 2007) to evaluate the mutagenic potentials of eight bleaching creams. The minimum inhibitory concentration (MIC) of each cream was ascertained to avoid the use of concentrations that might be toxic to the tester strain. MIC values ranging from 1mg/ml for ethidium bromide, Movate and Amos white to 4mg/ml for Tura, Maxitone, Top gel and Ultra clair were obtained. The MIC (2mg/ml) value obtained for Body white and Fair and white was almost as low as that obtained for ethidium bromide which was used as positive control and is a well known intercalating agent (mutagen). This may imply that Movate, Amos white, Body white and Fair and white creams might be better mutagens than Tura, Maxitone, Top gel and Ultra clair and may also act by intercalating in between stacks of base pairs of DNA.

When wild type *E. coli* (0157:H7) was exposed to each of the eight bleaching creams (in absence of S9 mix), there was alteration in as many as six (out of nine) morphological and biochemical characteristics of the wild type. With the criteria stated above, it implies that all the eight bleaching creams are mutagenic and can cause skin cancer in users since 90% correlation exist between mutagenicity and carcinogenicity (Ames *et al*., 1975). Little or no data exists in this part of the world regarding mutagenicity of bleaching creams in bacterial systems. The modified Ames test used in this study, has been similarly used by other researchers in the assessment of the mutagenicity of food additives (Akintonwa *et al*., 2007), some commonly used insecticides (Akintonwa *et al*., 2008), pharmaceutical industrial effluent (Akintonwa *et al*., 2009a), some medicinal plants (Akintonwa *et al*., 2009b) and crude oil fractions (Awodele *et al*., 2010).

There was almost a total growth inhibition (from full growth to scanty growth) when the test strain was treated with Body white, Tura, Movate, Amos white and Fair and white creams as was observed with ethidium bromide (positive control). Genetically, a forward and lethal mutation (from prototrophy to auxotrophy) may have occurred in these cases and have resulted in death of the tester strain. This has also demonstrated the antibacterial effect of each bleaching cream. Forward mutation may have occurred during lactose fermentation in Maxitone, Top gel, Body white, Movate , Amos white and Fair and white revertant, where this ability was gradually and completely lost. Treatment with Maxitone resulted in no phenotypic change. Full growth was observed as in the wild type. This could be as a result of silent mutation. In silent mutation, if a codon is affected, its coded function may not be lost due to code degeneracy in which another codon codes for the lost function and the cell survives. Silent mutations seem to have occurred also during lactose fermentation in Tura (TR1), Top gel (TGR1) and Ultra clair (UR1) auxotroph. The introduction of S9 mix gave no significant difference ($p > 0.05$) in the number of biochemical properties altered by each of the bleaching creams.

This implies that these bleaching creams are composed of potent mutagenic chemical ingredients

that do not need metabolic activation to exert their mutagenic effects. However, the introduction of S9 mix, exhibited back mutation (from scanty growth to full growth) in TR2, UR2 and TGR2, forward mutation (from scanty growth to complete absence of growth) in BR2, AR2 and FR2 and a silent mutation (from full growth to full growth) in MR2.

The mutagenic activity shown by the eight bleaching creams may not be unconnected with their chemical constituents as outlined in Table 1. Body white, Amos white and Fair and white creams consistently demonstrated higher mutagenic activity by their low MIC values and lethal effect on the bacterial cells. These three creams contain hydroquinone, among other depigmenting agents. Hydroquinone, according to Radhakrishnan *et al.* (2007) has carcinogenic properties and its use has been banned or limited in cosmetic products in many countries. Triethanolamine, a depigmenting agent (contained in Body white, Ultra clair, Top gel and Fair and white) has been reported to cause "fish odour" syndrome in users (Ruocco and Florio, 1995). Mahe *et al.* (1994) reported that the chemical, paraben (contained in almost all the creams) displayed estrogenic activity in several tests and can cause endocrine – disrupting action in users. Also warnings have been issued concerning paraben because of the possible link with breast cancer and reproductive effects in males.

Since the genetic material (DNA) in all living organisms (prokaryotic and eukaryotic) are based on the same structure, the result of this study implies that the eight bleaching creams can be genotoxic to body cells. It is therefore, recommended that their use should be avoided, except when prescribed by a physician. Government agencies such as NAFDAC and Consumer Protection Agency should embark on aggressive anti-bleaching campaign and awareness programmes to educate the public on the adverse effects of skin bleaching. The public should also be enlightened on the need to change the perception that "fairness is beauty" and made to understand that by the practice of good personal hygiene, good diet and healthy life style, it is possible for the skin to look good and radiant.

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